REMARKS

The allegation that the claims lack written description is traversed. Claim 1 has been amended to recite, *i.a.*, that the glycoprotein comprises at least one section of a human amino acid primary structure of CD55. Support for this amendment is found in the specification, *e.g.* on page 5, lines 12-18. Thus, the claims literally cover naturally occurring sequences and the recited glycostructure which reacts with SC-1. Thus, the Section 112 rejection should be withdrawn.

As for the anticipation rejections, neither Medof nor Tsuji, "as evidenced by Hensel," anticipates the instant claims. The Examiner improperly alleges that the proteins of the references possess "the inherent property of binding the SC-1 monoclonal antibody." There is no suggestion in the references that this alleged "inherent property" actually exists. Contrary to the assertion of the Examiner, it is the burden of the *PTO* to prove that the existence of such an allegedly "inherent property," is "reasonable" to presume. Only then does the burden shift to the applicant to disprove such an assertion. *In re Best*, 562 F.2d 1252, 195 USPQ430 (CCPA 1977).

Furthermore, applicants do not understand what is meant by the Examiner's statement "as evidenced by Hensel." The fact is that it is known the glycoproteins of the prior art do *not* exhibit the property of binding to the SC-1 monoclonal antibody. In the Medof ('84) reference cited by the Examiner in the Office Action, a glycoprotein is isolated from *non-tumor* cells (human red blood cells), which has a molecular weight of 70,000 D. Hensel does not disclose that this protein is "inherently" reactive toward SC-1. Rather, the proteins studied by Hensel are from *tumor* cells. Hensel does not disclose that SC-1 reacts with non-tumor cells, such as the red blood cells studied in the Medof ('84) reference. All this evidence supports only that Medof's protein is <u>different</u> from that of Hensel. Nothing supports the examiner's presumption.

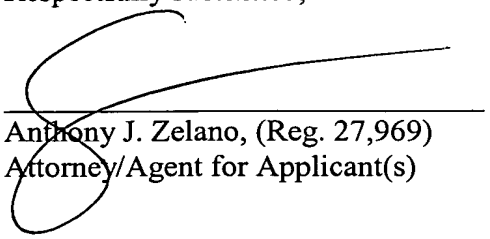
In a 1987 publication (Medof *et al.* (1987) *J. Exp. Med.*, 1987 March; 165(3): 848-64, attached), abstract, Medof and colleagues further characterize the so-called membrane-bound "wild type form" of CD55/DAF protein (70/72 kD) reported in their 1984 paper, and show that it is found not only on erythrocytes, but also on other non-tumor (non-transformed) cells, such as endothelium, epithelium, mucosa, etc. and on cell lines such as HeLa. In a more recent publication (Hensel *et al.* (2001, *Lab. Invest.*, Nov. 8(11): 1553-63, attached), this group clearly shows that SC-1 (which is shown to bind to the CD55/DAF, 82 kD, variant molecules as in the present invention) does *not* bind to HeLa cells. See, *e.g.*, pages 857-858. This reference also reports that the tumor cells 23132 express both types of CD55/DAF, the "Medof-type," having a molecular weight of 72 kD, and the type of the instant invention, having a molecular weight of 82 kD. Only the latter type of glycoprotein interacts with SC-1. These biochemical and immunochemical data prove unambiguously that the SC-1 antibody does not bind to the Medof CD55/DAF variant, but binds exclusively to a tumor-specific variant.

Similarly, the glycoprotein of Tsuji, also obtained from non-tumor human blood, does not "inherently" comprise a tumor-specific glycostructure that interacts with SC-1.

In view of the preceding amendments and arguments, the application is believed to be in condition for allowance, which action is respectfully requested.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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IDENTIFICATION OF THE COMPLEMENT DECAY-ACCELERATING FACTOR (DAF) ON EPITHELIUM AND GLANDULAR CELLS AND IN BODY FLUIDS

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A glycoprotein that inhibits complement activation is present on the surface of human erythrocytes (E^{hu})[1] (1–3). This ~70 kD surface component (3), termed decay-accelerating factor (DAF), interacts with autologous C4b and C3b that inadvertantly become associated with E^{hu} and prevents the covalently bound fragments from serving as sites for the uptake and conversion of C2 and factor B into enzymatically active C2a and Bb (4, 5). The interference by DAF with these C4b- and C3b-dependent cleavages prevents the assembly of C3 and C5 convertases of both complement pathways, i.e., all amplifying enzymes of the complement cascade (5). Unlike the serum regulatory proteins, C4 binding protein (C4bp) and factor H, which interact extrinsically with convertases on targets of complement (e.g., microorganisms or immune complexes), DAF acts only within the surface of the same cells, i.e., it is strictly an intrinsic membrane inhibitor (5). The ability of DAF to move freely in the plane of host E^{hu} membranes and efficiently restrict formation of autologous amplification convertases on their surfaces protects E^{hu} from injury that could arise from C4b or C3b deposited directly, e.g., via autoantibody, or in a bystander fashion as a result of complement activation in their vicinity.

Studies using flow cytometry and radioimmunometric assays based on anti-DAF antibodies (6, 7) have shown that DAF is present not only on the surface of E^{hu} but also in the membranes of circulating neutrophils, monocytes, B and T lymphocytes, and platelets, as well as on vascular endothelium (8), all cell types that are in intimate contact with serum complement proteins. In blood cells, DAF levels are highest in neutrophils and monocytes, cell types that interact closely with complement-bearing targets during the events that preceed their ingestion (6). Incorporation of exogenous DAF in vitro into affected E^{hu} of patients with paroxysmal nocturnal hemoglobinuria (PNH) (9), a disorder in which DAF is deficient (4, 6, 10–12), diminishes the exaggerated uptake of C3b that characterizes the complement-sensitive PNH E^{hu} in vivo (reviewed in reference 13). These findings that DAF deficiency is causally involved in the complement sensitivity of affected PNH cells have established that DAF activity in blood cells is essential under physiological conditions.

The present study was undertaken to investigate the distribution of DAF outside the vascular space.

This work was supported in part by grants from the Arthritis Foundation, the American Diabetes Association, the Systemic Lupus Erythematosus Society, and National Institute of Health grants AI-13224, AI-22800, and E-406337. M. E. Medof is an Investigator of The Arthritis Foundation. This work was presented in part at the 11th International Complement Workshop, Key Biscayne, FL (November 1985). M. E. Medof's and E. I. Walter's present address is Dept. of Pathology, Case Western Reserve University, Cleveland, OH 44106; J. Rutger's present address is Dept. of Pathology, Massachusetts General Hospital, Boston, MA 02114.

[1] *Abbreviations used in this paper:* A, rabbit hemolysin; AChE, acetylcholinesterase; Az, azide; C4bp, C4 binding protein; DAF, decay-accelerating factor; E^{hu}, human erythrocytes; E^{sh}, sheep erythrocytes; GVB^{++}, veronal buffer with gelatin; mfVSG, membrane form variant surface glycoproteins; PNH, paroxysmal nocturnal hemoglobinuria; PPO, 2,5-diphenyloxazole; PI-PLC, phosphatidylinositol-specific phospholipase-C; SFU, site forming unit; sVSG, soluble form of VSG.

Materials and Methods

Proteins, Antibodies, and Radiolabeling. E^{hu} DAF was purified from NP-40 extracts of stroma as described (5). The product appeared homogeneous upon analysis by SDS-PAGE and silver staining (see Fig. 1 of reference 5). Guinea pig C1 (14) and C3-9 (14) and human C4 (15), C2 (16), and C4bp (17) were purified as described. Murine anti-human DAF mAbs 1A10, 1IH6, and VIIIA7 (6); anti-human C3b/C4b receptor (CR1) mAbs 57F, 44D, and 31D (18); and antimalaria mAbs 2D3, 2A10, 3D11, and 6G2 (19) were obtained as described. Each was purified from ascites fluid by ammonium sulfate precipitation, DEAE-Sephacel chromatography, and Sephadex G200 gel filtration. Peroxidase-labeled goat anti-murine Ig (affinity purified, IgG and IgM H and L chain specific) was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Protein A–Sepharose was from Pharmacia Fine Chemicals (Piscataway, NJ).

Proteins and antibodies were labeled with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Iodinated products were purified by gel filtration followed by extensive dialysis.

Immunohistochemical Studies. Autopsy tissues and corneas (Oregon Lions Eye Bank, Portland, OR) preserved in RPMI medium were placed in OCT compound (Miles Laboratories Inc., Naperville, IL) and snap frozen. Cryostat tissue sections were fixed by sequential immersion for 2 min in cold acetone and 7 min in acetone/chloroform (1:1). After drying and washing three times with 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS), the fixed sections were overlayered with a predetermined optimal concentration (see Results) of pooled anti-DAF mAbs (1A10, 1IH6, and VIIIA7) in PBS containing 10% goat serum or with corresponding amounts of pooled anti-CR1 mAbs (57F, 44D, 31D) or nonrelevant antimalaria mAbs (2D3, 2A10, and 3D11) of the same subclasses in the same buffer. The antibody-treated sections were incubated in a moist chamber at 20°C for 1 h, and after thorough washing three times in PBS, they were overlayered a second time with a 1:40 dilution in PBS of peroxidase-labeled goat anti-murine Ig antibody. After another incubation at 20°C for 30 min and a second thorough washing in PBS, the sections were stained by immersion for 10 min in 50 ml of PBS containing 500 μl of 3,3′-diaminobenzidine and 150 μl of 3% hydrogen peroxide. Stained sections were washed in PBS, counterstained for 90 s with hematoxylin, and after extensive washing with cold water and dehydration by baths in absolute ethanol and xylene, they were mounted in Permount.

Affinity Purification of Urine DAF. Fresh urine voidings were individually collected into 500-ml polystyrene bottles containing 5 ml of 100 mM PMSF, 100 μg/ml leupeptin, 1.2 mg/ml soybean trypsin inhibitor, 100 μg/ml aprotinin, 200 mM benzamidine, 500 mM EDTA, and 0.5% sodium azide, and after mixing were immediately frozen at −70°C. When sufficient numbers of bottles from a given individual (usually 8–10) were accumulated, they were simultaneously thawed, their contents pooled, and the pooled urine was concentrated to 10% of the original volume in an Amicon Corp. (Danvers, MA) DC-2 hollow fiber apparatus equipped with an HIP30 cartridge. After dialysis against 150 mM NaCl, 25 mM Tris, 20 mM EDTA, pH 7.4 and clarification by centrifugation at 10,000 *g* for 60 min, the urine concentrate was applied to an affinity column composed of anti-DAF mAb 1A10 coupled to CNBr-Sepharose and crosslinked with glutaraldehyde (~100

ml concentrate/5 mg antibody) that was preequilibrated in the same buffer. After loading, the column was washed with 10 volumes of 0.5 M NaCl, 25 mM Tris, 20 mM EDTA, pH 7.4 and then eluted with 0.05 M diethylamine, 140 mM NaCl, pH 11.5 (20). Column fractions (usually 0.5 ml) were immediately neutralized by collection into tubes containing 0.1 ml of 1.0 M Tris, pH 6.0, saturated with glycine. Each fraction was assayed for DAF antigen by two-site radioimmunometric assay (see below) and positive fractions were examined by SDS-PAGE and silver staining. Urine DAF-containing fractions that were free of protein contaminants were pooled, dialyzed against PBS, and frozen in aliquots at −70°C. Overall yield varied from 20–100 μg urine DAF/L urine. The product gave a single band on Western blots developed with anti-DAF monoclonals and on autoradiographs after SDS-PAGE of the ^{125}I-labeled product.

Radioimmunometric and Hemolytic Assays. Quantitations of DAF concentrations by two site radioimmunometric assay were performed as described in (6). Briefly, samples and DAF standards were added in duplicate 25-μl aliquots to the wells of 96 well U-bottomed plastic microtiter plates (Becton Dickinson & Co., Oxnard, CA) precoated for 2 h at 20°C with 50 μl of 20 μg/ml anti-DAF mAb 1A10 and the wells were blocked with PBS containing 1% BSA. After incubation at 20°C for 2 h and washing three times with the blocking buffer containing 0.05% Tween-20, 25 μl of ^{125}I-labeled anti-DAF mAb IIH6 (containing ~10^5 cpm) was added, and after further incubation at 20°C for 1 h and extensive washing, bound DAF antigen in the samples was quantitated by comparison of counts in cut-out wells that received samples to those in wells that received DAF standards.

Hemolytic assays were performed as described (5) using complement intermediates prepared from sheep erythrocytes (E^{sh}) sensitized with 300 site forming units (SFU) of rabbit hemolysin (A) per cell. E^{sh}AC14 were prepared by incubating E^{sh}A sequentially with 300 SFU of guinea pig C1 and 10 SFU of human C4. E^{sh}AC142 were prepared by incubating the resulting E^{sh}AC14 with sufficient human C2 to yield cells bearing one hemolytic site of C4b2a after washing and decay for 15 min at 30°C. Incubations of E^{sh}AC142 with E^{lim} DAF and urine DAF were performed at 30°C in 145 mM NaCl, 2.5 mM veronal, pH 7.3, containing 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1% gelatin (GVB^{2+}). C4 hemolytic sites were quantitated by incubation of E^{sh}AC14 with 300 SFU of C2 followed by 300 SFU of guinea pig C3-9 and C4b2a sites developed by direct incubation of E^{sh}AC142 with 300 SFU of guinea pig C3-9.

SDS-PAGE and Western Blotting. SDS-PAGE was conducted on 7.5% linear slab gels according to the method of Laemmli (21). Gels of ^{35}S-labeled samples were washed with DMSO and treated with 2,5-diphenyloxazole (PPO) followed by water before drying. Autoradiography and fluorography were performed at −70°C on X-Omat XAR-5 film (Eastman Kodak Co., Rochester, NY).

For Western blots, proteins were subjected to SDS-PAGE on 7.5% gels under nonreducing conditions. The separated proteins were transferred to nitrocellulose using a Transblot Apparatus (Bio-Rad Laboratories, Richmond, CA). After blocking for 1 h at 37°C with PBS containing 5% BSA and 0.05% azide (AZ) (PBS-BSA-AZ), nitrocellulose strips were incubated at 20°C for 1 h with ^{125}I-labeled anti-DAF mAb IIH6, washed three times with PBS-BSA-AZ, dried, and loaded into film cassettes.

Preparation of Cell Extracts and Immunoprecipitation. For radioimmunometric assays, cell monolayers were washed with PBS and extracted for 20 min at 0°C with 50 μl/10^6 cells of 0.5% NP-40 in PBS containing 1 mM PMSF and 5 mg/ml synthetic elastase inhibitor [Suc-(OMe)-Ala-Ala-Pro-Val-MCA] (Peninsula Laboratories, Inc., Belmont, CA). The NP-40 extracts were centrifuged at 12,000 *g* for 15 min and the supernatant was transferred to new tubes. For SDS-PAGE and Western blot analyses, cell monolayers were scraped from culture dishes with 2% SDS in 20 mM Tris, pH 7.5, 100 μ/ml trasylol (250 μl/10^6 cells) using a rubber policeman. The SDS extracts were boiled for 10 min, diluted fivefold with 50 mM Tris, pH 7.4, containing 190 mM NaCl, 100 μ/ml trasylol, 6 mM EDTA, and 2.5% Triton X-100 (Tris-NaCl-EDTA), the mixture was centrifuged at 10,000 *g* for 15 min, and the supernatant was transferred to a new tube.

Samples of SDS-cell extracts (usually 600 μl) or of extracellular fluids or culture supernatants (200–4,000 μl) were preabsorbed for 2 h at 20°C with 100 μl of 10% Protein A–Sepharose in Tris-NaCl-EDTA and were centrifuged. Supernatants were transferred to new tubes, pooled anti-DAF mAbs 1A10, IIH6, and VIIIA7 (5 μg/ml each) or nonrelevant antimalaria mAbs (of the same subclasses, 5 μg/ml each) were added, and the mixtures were rotated for 2 h at 20°C. Immune complexes were precipitated by addition of 100 μl of fresh 10% Protein A–Sepharose in Tris-NaCl-EDTA buffer, further rotation for 1 h at 20°C, and centrifugation. The beads were transferred to new tubes, washed twice with Tris-NaCl-EDTA buffer, and immunoprecipitated proteins were extracted by addition of 50 μl of SDS-PAGE sample buffer followed by boiling for 3 min.

Biosynthetic Studies. Biosynthetic labeling was performed as described (22). Semiconfluent (60–70%) HeLa cell monolayers were prepared in 60-mm culture plates. The cells were preincubated for 1 h before labeling with cysteine (Cys)-free RPMI containing 10% dialyzed FCS. [^{35}S]Cys (50 μCi; New England Nuclear, Boston, MA) was added in 100-μl aliquots to replicate plates. The plates were placed at 37°C, and after various times, the plates were transferred to ice, the medium was removed, and the cells were extracted with 2% SDS in Tris buffer as described above.

Results

Expression of DAF by Epithelial Cells. To determine whether DAF is expressed by cell types other than those in the vascular space, immunohistochemical analyses of various tissues were performed with anti-DAF mAbs. Since B lymphocytes express 7 × 10^4 DAF molecules per cell (6), initial studies were carried out with lymph node specimens to establish optimal conditions for the detection of DAF in tissue sections. Sections of the lymph nodes (fixed as described in Materials and Methods) were incubated with serial dilutions of pooled anti-DAF monoclonals, corresponding dilutions of pooled anti-CR1 mAbs, or with nonrelevant (antimalaria) mAbs as controls. After washing and development with peroxidase-labeled goat anti-mouse Ig (see Materials and Methods), the sections were examined by light microscopy. Bright anti-DAF staining of germinal center lymphocytes was observed after treatment of the sections with 250 ng/ml of pooled anti-DAF mAbs, while no staining was observed with 10 μg/ml of nonrelevant mAbs. The anti-DAF staining was most intense in lymphocytes also stained by anti-CR1 mAbs (100 ng/ml) but was also apparent in lymphocytes that were anti-CR1–negative.

Samples of different tissues were next collected from surgical and autopsy specimens, frozen sections of the specimens were fixed and stained, and the sections were examined as described above for the lymph nodes. Selected photographs of the results are shown in Figs. 1 and 2. Unexpectedly, strong anti-DAF staining of epithelial cells in multiple locations was observed. The anti-DAF staining of epithelium was striking in cornea (Fig. 1*A*), conjunctiva (Fig. 1*B*), oral mucosa and salivary glands (Fig. 1*C*), esophagus (Fig. 2*A*), upper and lower GI tract (not shown), and in kidneys and urinary tract (not shown). The anti-DAF staining was also prominent in endocardium (not shown), synovial cells (Fig. 2*B*) lining joint spaces, and in endometrial and epithelial cells lining uterus (Fig. 2*C*) and cervix (not shown). In kidneys, epithelium was positive in proximal tubules, descending and ascending loops of Henle and in collecting ducts. In contrast to previously reported findings (23, 24) and control studies with anti-CR1 antibodies, anti-DAF staining in renal cortex was not localized to glomeruli (podocytes) but was diffuse. In the urinary tract, urethelium was strongly positive in medullary spaces, ureter, bladder, and urethra (not shown). In all sites with layered epithelium, intensity of anti-DAF staining increased with cell maturation

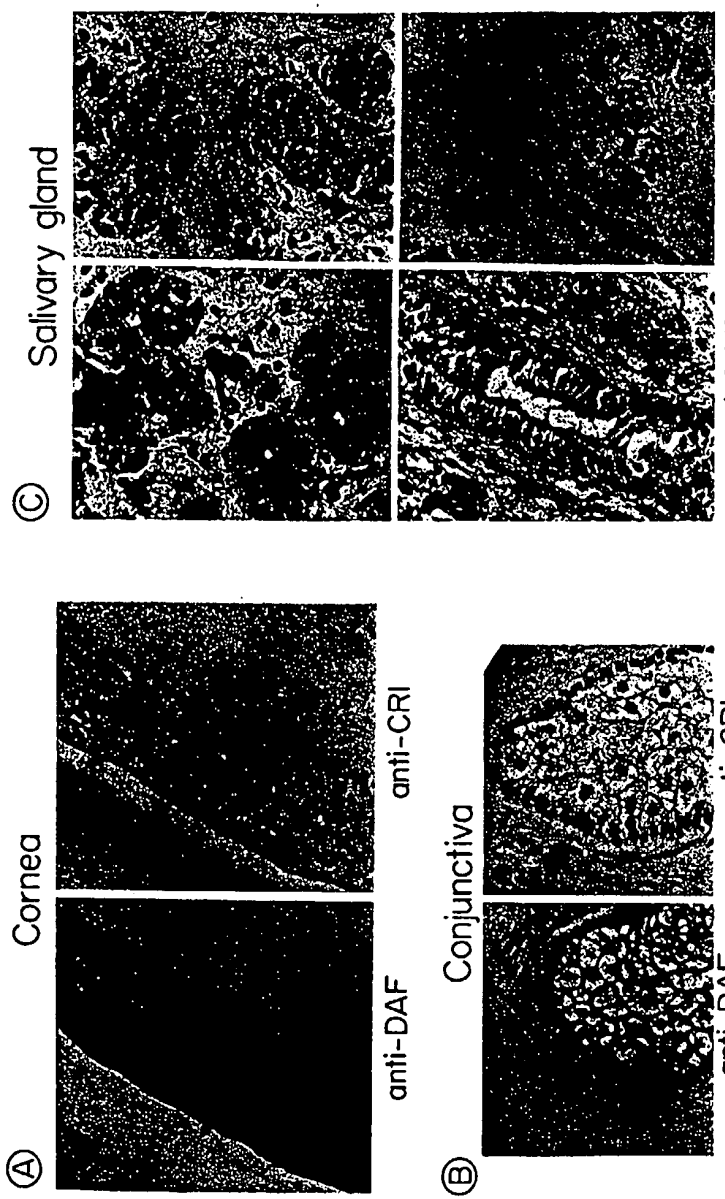

FIGURE 1. Various tissues were examined immunohistochemically for DAF using anti-DAF mAbs and the peroxidase method. Anti-DAF and control anti-CR1-stained sections of tissues are shown on the left and right hand sides of each panel, respectively. (*A*) Marked anti-DAF staining of corneal epithelium is apparent. The staining is localized to the epithelial cell membrane and increases in intensity in upper epithelial cell layers. (*B*) Sections of tarsal conjuctiva are shown. Prominent anti-DAF staining of glandular tissues is apparent. (*C*) Anti-DAF staining of salivary glands is observed. The staining is most intense on internal luminal surfaces.

and staining was greatest in epithelial margins and on surfaces.

In addition to the epithelial cell staining, intense anti-DAF positivity of exocrine gland cells in many sites (Figs. 1, *B* and *C*; 2*A*) was apparent and strong anti-DAF staining within extracellular matrix in several tissues (Figs. 1*B*; 2, *A* and *C*) was noted. As observed with epithelial cells, the intensity of anti-DAF staining of glandular cells increased with cell maturation. The anti-DAF staining within extracellular matrix consistently occurred in a fibrillar pattern. The fibrillar staining was prominent in fibrous sheaths surrounding myocardial muscle bundles, interstitium underlying endocardium, and connective tissue adjacent to synovium (Fig. 2*B*). The nature of the fibrils is unknown. Patterns of anti-DAF staining of epithelium, glandular cells, and connective tissue similar to those shown (in Figs. 1 and 2) were also observed in pleura, pericardium, and peritoneum.

Soluble DAF Forms in Extracellular Fluids. The possibility that DAF antigen might be present in extracellular fluids adjacent to epithelial cell surfaces surrounding various extracellular compartments was next investigated. Samples of different extracellular fluids were obtained and the specimens were assayed for DAF antigen by two-site radioimmunometric assay using anti-DAF mAbs.

The results of analyses of tears, saliva, synovial fluid, cerebrospinal fluid, and of plasma are summarized in Table I. DAF antigen was present in all fluids tested, and the concentrations were in most instances greater than that in plasma. Measurements of DAF in urine samples collected from 10 normal individuals are given in Table II. 75–500 ng/ml of antigen was found in spot specimens. Quantitations of antigen in 24-h urine collections revealed outputs as high as 500 μg, a value corresponding to ~3% of the total intravascular blood cell–associated membrane DAF.

To study the nature of DAF antigen present in the body fluids, the antigens detected in tears, saliva, and urine were precipitated with anti-DAF mAbs and examined on Western blots. The antigens in tears and saliva migrated with apparent M_r higher (>100,000) (not shown) than E^{hu} DAF, while that in urine migrated with a slightly lower M_r (~67,000) (see below). The more abundant urine antigen was isolated for further structural and functional investigations. Large volumes (~5 liters each) of urine from six normal individuals were collected into vessels containing multiple protease inhibitors. The six urine collections were separately concentrated, and the DAF antigen in each purified by affinity chromatography and compared by Western blot analysis (Fig. 3*A*). In all cases, the urine DAF variant was ~3 kD smaller than membrane DAF isolated from E^{hu}.

Urine DAF from one individual (Fig. 3*A*, lane *1*) was next tested in complement assays for functional activity and its regulatory properties were compared to those of E^{hu} membrane DAF. Unlike purified E^{hu} DAF, which incorporated into E^{sh}AC14 and markedly inhibited C4 hemolytic activity on washed E^{sh}AC14 intermediates (Fig. 3*B*), the urine DAF had little activity in this assay. However, when the urine molecule was incubated in the presence of E^{sh}AC142 bearing preformed C4b2a, it accelerated C4b2a decay with an efficiency comparable to that of serum C4bp. The decay-accelerating effect was blocked by addition of pooled anti-DAF mAbs. A simple interpretation of these findings is that urine

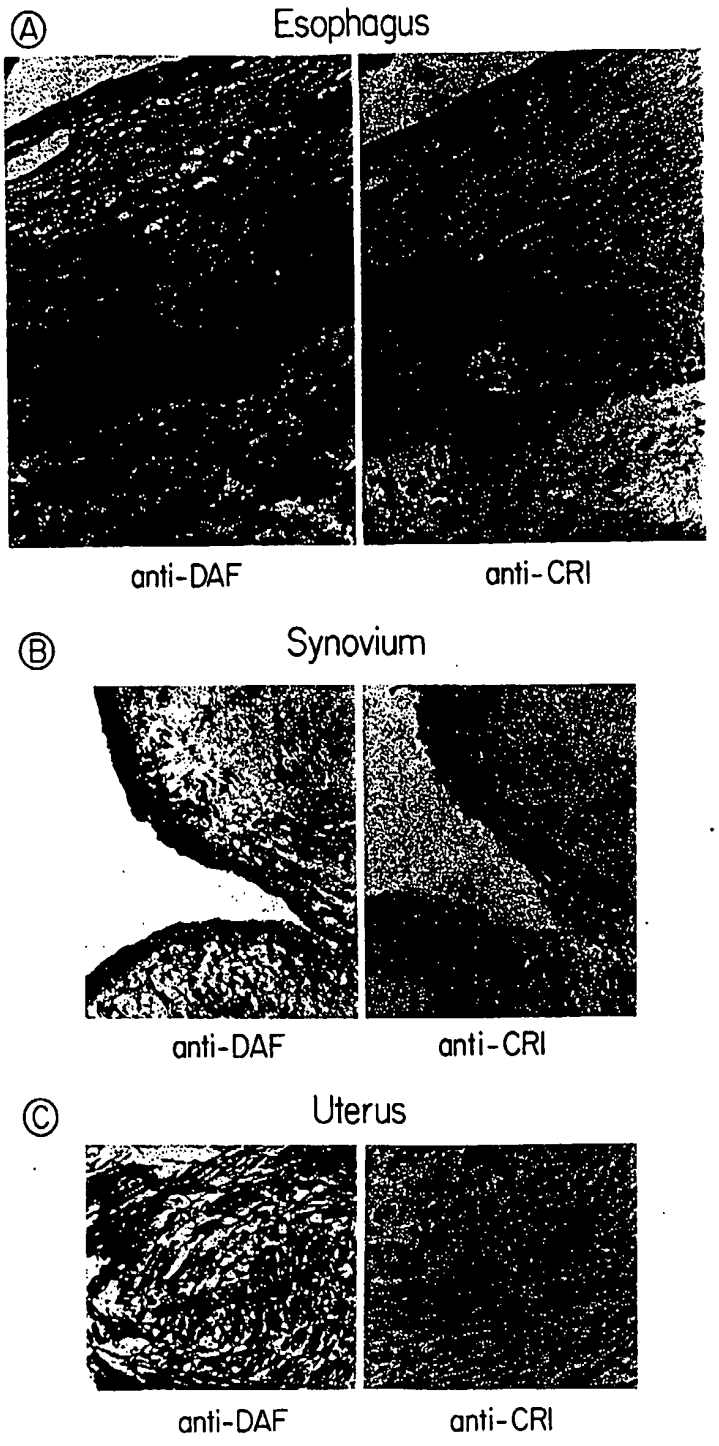

TABLE I
DAF Levels in Body Fluids

Fluid	Number of samples	DAF concentration ± SD
		ng/ml
Plasma	12	64 ± 26
Saliva	9	112 ± 51
Tears	2	344, 68
Synovial fluid	8*	168 ± 99
Cerebrospinal fluid	1‡	38

* Osteoarthritis.
‡ Evaluation for headache.

TABLE II
Levels of Urine DAF in Normal Individuals

Donor	DAF	Volume	Total DAF
	ng/ml	*ml/24 h*	*μg/24 h*
RW	150	1,000	150
AC	170	1,700	290
RC	127	1,600	200
RR	100	3,200	320
VN	200	2,550	510
AF	250	1,400	350
MD	350	1,200	420
RM	400	1,200	480
EW	75	1,700	128
SK	75	2,450	185
DE	200	1,200	240
Mean			298

DAF is unable to incorporate into cell membranes and therefore cannot exert its effect intrinsically. Indeed, urine DAF is less hydrophobic than membrane DAF. When subjected to chromatography on Phenyl-Sepharose beads, all of the membrane DAF bound tightly to the resin, while the urine molecule remained >90% in the fluid phase, indicating that it differs markedly with respect to hydrophobicity (not shown).

Appearance of a Soluble DAF Form Resembling Urine DAF from HeLa Cell and Foreskin Epithelium Culture Supernatants. In view of the concurrent findings of high levels of DAF expression by epithelial cells and the presence of soluble DAF forms in adjacent body fluids, in vitro studies were next performed to investigate the possible epithelial cell origin of the extracellular DAF species. For this purpose, human foreskin epithelium and the HeLa epithelial cell line were cultured.

FIGURE 2. (*A*) Anti-DAF staining of esophageal mucosa is evident. Prominent staining of glands and fibers in underlying submucosal connective tissue additionally can be appreciated. (*B*) Localized anti-DAF staining of synoviocytes and fibers is observed. Staining of fibers is again appreciated. (*C*) The staining of fibers underlying uterine endometrium is prominent.

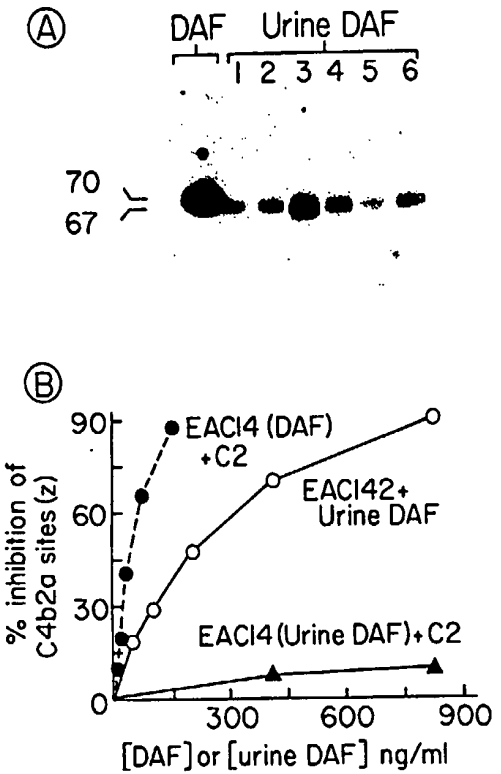

FIGURE 3. Comparison of the gel mobilities and functional properties of urine DAF and E^{hu} membrane DAF. (*A*) Urine DAF samples were immunoprecipitated from urine concentrates prepared from six normal individuals and compared with E^{hu} membrane DAF on Western blots after electrophoresis of proteins on 7.5% SDS-PAGE gels under nonreducing conditions. In the case of all six urine DAF isolates a single band ~3,000 smaller in apparent M_r than E^{hu} membrane DAF was observed. $M_r \times 10^{-3}$ shown at left. (*B*) Purified urine DAF and E^{hu} membrane DAF were incubated with E^{h}AC14, and after washing, residual C4b sites were developed by addition of C2 and C3-9. Alternatively, the urine DAF was incubated for 15 min at 30°C with E^{h}AC142 and remaining C4b2a sites were assayed by addition of C3-9. A control using urine DAF in the presence of anti-DAF mAbs was performed (not shown). Unlike E^{hu} membrane DAF, the urine DAF had no effect on E^{h}AC14 but it inhibited hemolytic activity of preformed C4b2a with efficiency comparable to C4bp.

As shown in Fig. 4, immunohistochemical analyses of both cultured cell types showed strong anti-DAF staining. Immunoradiometric assays of the HeLa cell extracts revealed ~2×10^5 DAF molecules per cell, approximately fourfold more DAF than present in polymorphonuclear leukocytes (6). Purification of HeLa cell DAF by immunoprecipitation with anti-DAF mAbs and Western blot analyses of the immunoprecipitate demonstrated that the HeLa epithelial cell DAF was similar in apparent size (M_r ~72,000) to blood mononuclear cell membrane DAF (see reference 6).

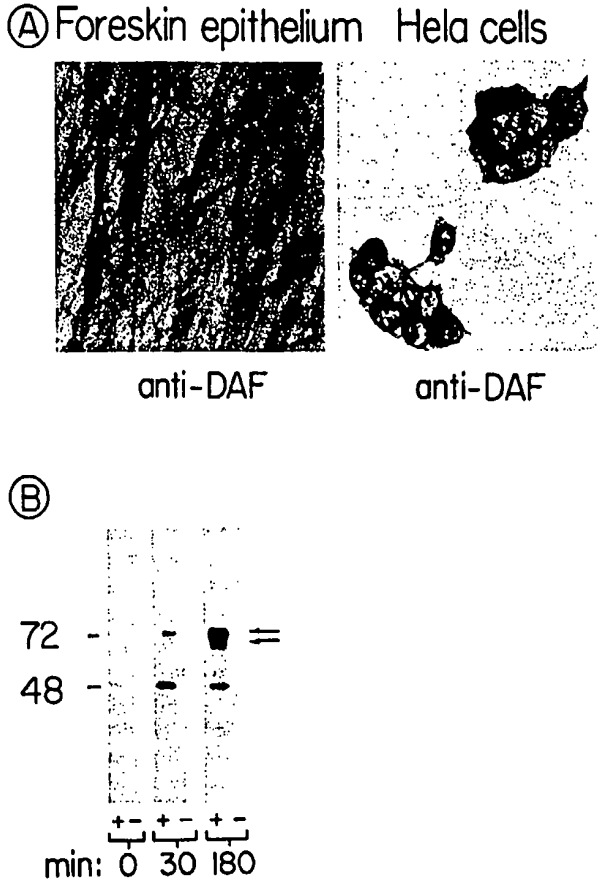

FIGURE 4. Human foreskin epithelium and the HeLa cell line were cultured. Immunohistochemical analyses of the cultured cells showed strong anti-DAF staining (*A*). Immunoradiometric assays of the HeLa cell extracts revealed ~2×10^5 membrane DAF molecules per cell. (*B*) Fluorographs of a continuous labeling study of DAF biosynthesis in HeLa cells. After a 1-h incubation with Cys-free RPMI and labeling with [^{35}S]Cys for the time periods shown, the monolayers were extracted with SDS and newly labeled DAF proteins were isolated by immunoprecipitation and analyzed by SDS-PAGE/fluorography. The lanes designated (−) show the results of control studies with nonrelevant mAbs. $M_r \times 10^{-3}$ shown at left.

HeLa cells were next cultured, and during log phase growth, the culture supernatant was analyzed at different times by radioimmunometric assay for DAF antigen. The results showed a progressive accumulation of the antigen in the culture medium (Fig. 5). In contrast, no DAF antigen was detectable in the supernatant of K562 erythroleukemia cells that also contain DAF.

HeLa cells were next cultured for various times in the presence of [^{35}S]Cys and newly synthesized DAF in the membranes of the cells and in the culture

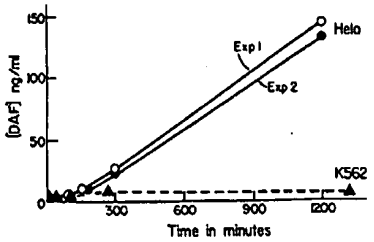

FIGURE 5. HeLa cells were cultured, and during log phase growth, the culture supernatant was assayed at different times for DAF antigen by radiometric assay. While no DAF antigen was detectable in the supernatant of DAF-containing (4×10^6 cells/ml) K562 erythroleukemia cells, progressively increasing levels of DAF antigen were measurable in the HeLa cell (2×10^6 cells/ml) supernatants.

supernatant were isolated by specific immunoprecipitation and analyzed by SDS-PAGE/radioautography. In addition to mature 72 kD HeLa membrane DAF and a 48 kD intracellular DAF precursor (22), a DAF species with apparent M_r 5,000 smaller than the mature membrane molecule was isolated from the cells. The larger but not the smaller DAF species was detectable on autoradiographs of SDS-PAGE gels of anti-DAF immunoprecipitates of ^{125}I surface-labeled HeLa cells. A DAF molecule of comparable size to the smaller DAF species was recovered from the culture supernatant (not shown). When examined next to urine DAF on Western blots, this 67 kD DAF molecule appeared similar in gel mobility.

Discussion

In previous studies, we and others showed that DAF is present on the surface of blood cells (6, 7) and vascular endothelium (8) where its activity is essential to protect these cell types from serum complement proteins. In the present study, we investigated the distribution of DAF in tissues and body fluids outside the vascular space. Immunohistochemical analyses revealed large amounts of DAF antigen on the surface of epithelium in multiple sites and radioimmunometric assays identified soluble forms of the antigen in several extracellular fluids. Purification of the most abundant extracellular DAF form, urine DAF, and analyses of its properties demonstrated that, like E^{hu} membrane DAF, it possessed C4bp-like complement regulatory activity. Unlike E^{hu} DAF, however, it was unable to incorporate into membranes and on SDS-PAGE appeared ~3 kD smaller in apparent size. Studies using primary cultures of foreskin epithelium and the epithelial cell line HeLa demonstrated *de novo* biosynthesis of DAF protein in amounts greatly exceeding those in blood cells. Analyses of the culture supernatants of these studies disclosed the release by these cell types of a soluble DAF form closely resembling urine DAF in SDS-PAGE gel mobility.

In our immunohistochemical analyses, epithelial cells stained brightly for DAF in all locations where surface epithelium is found. These included cornea, conjunctiva, oral and gastrointestinal mucosa, renal tubular epithelium and urinary tract urethelium, uterine endometrium and cervical epithelium, pleural and pericardial serosa, and synoviocytes lining diarthrodial joint spaces. In addition to the anti-DAF staining of these surface cells, strong anti-DAF staining of exocrine glandular cells was observed throughout many tissues. In the case of both surface epithelium and glandular cells, intensity of anti-DAF staining increased in cells closest to the surface. Within glands, the most intense staining was observed in lumens. The increase could represent induction of DAF expression with cell differentiation/maturation. In vitro studies with epithelial cell lines (reviewed in reference 25) have shown that expression of certain membrane proteins is polarized to the nonadherent exterior cell surface.

The physiologic function that DAF serves on epithelial and glandular cell surfaces is not yet apparent. The principal secretory immunoglobulin is IgA and it can only activate the alternative pathway of complement. However, other immunoglobulins, e.g., IgM, are found in smaller amounts in secretions, as are complement components (26). The biological significance of complement activation in secretions is not understood. It is possible that the membrane DAF functions to prevent complement-mediated injury to the epithelial and glandular cells in a fashion similar to its postulated role in cells found in the vascular space. Alternatively, it is possible that the epithelial and glandular cell DAF could have some other, noncomplement-related activity.

Our finding that in skin, joints, and uterus, DAF is associated with fibrils raises a number of questions. No staining in blood vessel walls, in cartilage, or of reticular or elastic fibers occurred. The staining pattern followed that of type I collagen fibers on sections counter-stained with Giemsa or Toluidine Blue, raising the possibility that the DAF-positive fibrils may contain type I collagens, which in different tissues are related but not identical (27). We cannot exclude the possibility that the staining represents a spurious crossreaction, although this is unlikely because various mAbs reacting with different DAF epitopes gave identical results. Interestingly, asymmetric forms of acetylcholinesterase (AChE), a protein that is structurally related to DAF (see below), are associated with the extracellular matrix at neuromuscular junctions and in fish electric organs. In Disopyge tchudii electric organ, an asymmetric form of AChE consisting of 12 catalytic subunits attached to a triple helical collagen-like tail is anchored noncovalently to the extracellular matrix through a linkage that appears to involve heparin sulfate proteoglycan (29). It is tempting to speculate that the association of DAF with collagen fibers that contain hydroxyl or amino groups able to condense with nascent C4b or C3b could serve to reduce the potential for complement-mediated damage in connective tissues.

The identification of large amounts of DAF antigen in various extracellular fluids by two-site radioimmunometric assay (Table I) was surprising. Since positivity in this assay depends on reactivity with two mAbs directed against different DAF epitopes, detection of the antigens by this method implied that they must contain a significant portion of the E^{hu} membrane DAF structure. Immunoprecipitation of urine DAF with anti-DAF mAbs and Western blot analysis showed that the antigen migrated with apparent M_r 3,000–8,000 smaller than membrane DAF of E^{hu} (Fig. 3*A*) and other blood cells (6, 7). The small difference in apparent size between this extracellular DAF form and blood cell DAF molecules was associated with a marked difference in hydrophobicity as shown by its lack of adsorption to Phenyl-Sepharose and inability to incorporate

into membranes (Fig. 3 *C*). The demonstration that urine DAF functioned (Fig. 3 *C*) similarly to the serum regulatory protein, C4bp, indicated that urine DAF was able to interact with the classical pathway C3-convertase and therefore contained a DAF functional site. The dissociation of membrane incorporation and convertase inhibitory activity observed in these studies thus implies that membrane anchorage and complement regulatory activity in membrane DAF are mediated by different structures.

Structural studies of E^{hu} membrane DAF have shown that it is an amphipathic protein (22, 30). Unlike most membrane proteins that contain polypeptide membrane anchors consisting of hydrophobic amino acids, E^{hu} membrane DAF possesses a glycolipid membrane anchor. This unconventional non–amino acid anchoring structure is linked covalently to the COOH-terminus of DAF polypeptide and is similar to anchoring structures that recently have been described in (membrane form) variant surface glycoproteins (mfVSGs) of trypanosome (31, 32) of leishmania (33) parasites, murine thymocyte Thy-1 antigen (34, 35), and E^{hu} AChE (36–38). It is composed of an oligosaccharide, containing ethanolamine(s) and nonacetylated-glucosamine, that is attached through the glucosamine glycosidic bond to a phospholipid composed of inositol and fatty acids (22). The fatty acids are inserted in the cell lipid bilayer and permit lateral mobility of the attached protein. The phospholipid can be partially cleaved by phosphatidylinositol-specific phospholipase-C (PI-PLC) (30, 22, 39). This cleavage releases membrane DAF from cells and generates a hydrophilic DAF derivative of slightly reduced apparent M_r similar to urine DAF.

Our studies with foreskin epithelium and HeLa cells (Fig. 4 *B*) verified that epithelial cells synthesize membrane DAF and additionally showed that this cell type releases a DAF form into cell culture supernatants (Fig. 5). Analysis of the released DAF molecule showed that it was ~5,000 smaller in apparent M_r than epithelial cell membrane DAF and similar in mobility on gels to urine DAF. This finding suggests that urine DAF may be synthesized by the adjacent urethelium. Other extracellular DAF forms could likewise be generated by adjoining epithelia and/or glandular cells. The soluble DAF forms could be products of different genes than those that encode membrane DAF molecules, or could arise from alternative processing of DAF mRNA occurring after transcription resulting in secreted rather than membrane forms. Studies with VSGs and Thy-1 antigen have shown that the cDNAs that code for these proteins predict COOH-terminal extension peptides of 15–31 amino acids that are not found in the mature proteins (40, 41). There is evidence from biosynthetic studies with VSGs, that the missing COOH-terminal peptide is excised and replaced with the mfVSG glycolipid anchor immediately after formation of VSG polypeptide on ribosomes (42, 43). Consistent with this notion, studies of DAF biosynthesis (22) have shown that anchor components are associated with 48 kD pro-DAF before processing of pro-DAF in the Golgi. The lack of incorporation of the membrane DAF glycolipid or the attachment of a different moiety to the DAF COOH-terminus could result in a soluble DAF form. Alternatively, the soluble DAF species could derive from membrane DAF molecules themselves. Soluble forms of VSGs, termed sVSGs, can be isolated from trypanosome cultures (31). The sVSGs resemble urine DAF in that they are similar in M_r to the corresponding mfVSGs

and markedly less hydrophobic. They have been shown to arise via the action of an endogenous PI-PLC present in trypanosome membranes (reviewed in references 44 and 45). Structural analyses of the soluble DAF forms for components of the membrane DAF glycolipid anchor proximal to the known site of PI-PLC cleavage could clarify whether the soluble DAF forms arise by a similar mechanism.

Summary

Decay-accelerating factor (DAF) is a 70 kD membrane regulatory protein that prevents the activation of autologous complement on cell surfaces. Using immunohistochemical methods and a radioimmunometric assay based on mAbs to DAF, we found large amounts of membrane-associated DAF antigen on the epithelial surface of cornea, conjunctiva, oral and gastrointestinal mucosa, exocrine glands, renal tubules, ureter and bladder, cervical and uterine mucosa, and pleural, pericardial and synovial serosa. Additionally, we detected soluble DAF antigen in plasma, tears, saliva, and urine, as well as in synovial and cerebrospinal fluids. While plasma, tear, and saliva DAF are larger than erythrocyte (E^{hu}) membrane DAF by Western blot analysis, urine DAF is slightly smaller (67,000) in M_r. Unlike purified F^{hu} DAF, however, urine DAF is unable to incorporate into the membrane of red cells. Although its inhibitory activity on the complement enzyme C3-convertase is lower than that of E^{hu} DAF, it is comparable to that of serum C4 binding protein (C4bp). Biosynthetic studies using cultured foreskin epithelium and Hela cells disclosed DAF levels (~2×10^5 molecules/cell) exceeding those on blood cells. In addition, these studies revealed the synthesis of two DAF species, one with apparent M_r corresponding to that of epithelial cell membrane DAF and the other to urine DAF, suggesting that the urine DAF variant arises from adjacent epithelium. The function of DAF in body fluids is unknown, but the observation that urine DAF has C4bp-(or factor H–)like activity shows that it could inhibit the fluid phase activation of the cascade.

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SCHISTOSOMA MANSONI SHARES A PROTECTIVE CARBOHYDRATE EPITOPE WITH KEYHOLE LIMPET HEMOCYANIN

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The production of mAbs of various isotypes to *Schistosoma mansoni* has greatly contributed to the appreciation of the function of antibodies in the immunity against schistosomiasis, and has allowed the characterization of several potential protective antigens. Using this approach, we clearly established in previous studies (1, 2) the close relationship existing between a 38,000 M_r *S. mansoni* schistosomulum surface antigen and the expression of eosinophil-mediated killing of schistosomula. The 38,000 M_r antigen was initially characterized by the IPLSm1 rat IgG2a mAb that exhibits a marked eosinophil-dependent cytotoxicity and passively transfers a high degree of protection towards a cercarial challenge (1, 2). This particular surface antigen was also shown to react with polyclonal antibodies present in various infected hosts including mice, rats, monkeys, and humans (3). Additional studies revealed that 97% of a group of 120 Brazilian patients with *S. mansoni* infection produced circulating antibodies against this antigen, suggesting that the 38,000 M_r molecule corresponds to a potent immunogen in man. More interestingly, the antibody response was demonstrated to appear in young children, to be maximal in older patients, and showed a parallelism with the prevalence and the intensity of the infection. This indicated that the antibody response against the 38,000 M_r antigen could be considered an important marker of *S. mansoni* infection (4).

Although the 38,000 M_r antigen initially appeared as a good candidate for the molecular cloning and subsequent studies, the glycanic nature of the epitope recognized by the IPLSm1 mAb limited its in vitro production by recombinant DNA methodology. Secondly, the 38,000 M_r antigen was shown (5) to bind the IPLSm3 mAb of IgG2c isotype capable of blocking both in vivo and in vitro effector function(s) of the IPLSm1 IgG2a mAb. We recently demonstrated (6) the existence of similar blocking antibodies specific to the 38,000 M_r molecule

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Regulation of the New Coexpressed CD55 (Decay-Accelerating Factor) Receptor on Stomach Carcinoma Cells Involved in Antibody SC-1–Induced Apoptosis

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SUMMARY: The human monoclonal antibody SC-1 was isolated from a patient with a diffuse-type adenocarcinoma of the stomach using somatic cell hybridization. The immunoglobulin (Ig)M antibody reacts specifically with diffuse- (70%) and intestinal-type (25%) gastric adenocarcinoma and induces apoptosis in vitro and in vivo. When used in clinical trials with stomach carcinoma patients, significant apoptotic and regressive effects in primary tumors have been observed with the antibody SC-1. The SC-1 receptor is a new 82 kd membrane-bound isoform of glycosylphosphatidylinositol (GPI)-linked CD55 (decay-accelerating factor, DAF). CD55 is known to protect cells from lysis through autologous complement and is coexpressed with the ubiquitously distributed 70 kd isoform. The SC-1–specific CD55 isoform is up-regulated shortly after antibody binding, followed by an internalization of the antibody/receptor-complex, whereas the membranous expression of wild-type CD55 remains unchanged. The apoptotic process is marked by cleavage of cytokeratin 18, indicating the involvement of caspase-6 in the apoptotic process. In contrast to other apoptotic pathways, a cleavage of poly(ADP-ribose)polymerase (PARP) is not observed. The expression of the cell-cycle regulator c-myc becomes up-regulated, whereas expression of topoisomerase IIα is down-regulated. Induction of apoptosis leads to an increase in the internal Ca^{2+} concentration, which is not necessary for the apoptotic process but for the transport of newly synthesized SC-1–specific CD55 isoform to the membrane. (*Lab Invest 2001, 81:1553–1563*).

Malignant cells re-express, mask, or modify surface structures to fulfill requirements for a higher proliferation rate, to escape immune response mechanisms, or just through happenstance. However, in most cases, these "new" structures allow the immune system to recognize, attack, and remove transformed cells at early stages. Manifest tumors are therefore not the result of a missing qualified immune response, but instead a matter of quantity. Cancer patients represent an enormous source of tumor-specific and -reactive reagents, such as cells, factors, and antibodies. Human hybridomatechnology offers an ideal tool for isolating and establishing human tumor-specific antibodies for therapy and diagnosis.

We have recently described the human antibody SC-1, isolated from a patient with a signet ring cell carcinoma of the stomach (Vollmers et al, 1989). This immunoglobulin (Ig)M antibody induces apoptosis of gastric cancer cells in vitro and in vivo and is being used successfully in clinical trials (Vollmers et al, 1998b). The receptor of SC-1 was found to be a modified version of CD55 (decay-accelerating factor, DAF). This protein protects host tissues from autologous complement activation and is expressed on all cell types that are likely to have contact with the complement system, ie, epithelial cells (Koretz et al, 1992), lymphocytes, monocytes, platelets (Nicholson-Weller et al, 1985), and endothelial cells (Asch et al, 1986). It acts by dissociating the classical and alternative pathway C3 convertases, and there are various isoforms in existence (Lublin et al, 1986). CD55 is expressed in two different isoforms (DAF-A and DAF-B) generated by differential splicing. Whereas DAF-A is secreted from cells, DAF-B is linked to cells by a glycosylphosphatidylinositol (GPI) anchor (Caras et al, 1987). Both forms are further modified by different glycosylation patterns, resulting in molecular weight sizes from 55 to 100 kd (Hara et al, 1993).

Besides these well-described functions of CD55, it has become more and more obvious that this receptor can also act as a signal-transducing molecule. With monoclonal antibodies directed against CD55, human monocytes can be activated in vitro (Shibuya et al, 1992). These changes in the cell cycle might be transmitted through src-kinases, which are associated with the GPI anchor of CD55 (Parolini et al, 1996; Shenoy-Scaria et al, 1992).

Another aspect of CD55, which makes it interesting as a target for tumor therapy, is the fact that this

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molecule is overexpressed on various tumors. Overexpression has been found in, eg, breast, colon, and stomach carcinoma (Hofman et al, 1994; Koretz et al, 1992; Niehans et al, 1996), and this overexpression makes CD55 a suitable target for cancer vaccines in the treatment of colon carcinoma (Spendlove et al, 1999). Vaccination with a human anti-idiotype antibody that mimics CD55 was used for adjuvant treatment of colon carcinoma and resulted in activation of a cellular anti-tumor response (Durrant et al, 1995, 2000). However, this overexpression of CD55 and other complement-inactivating molecules limits therapeutical approaches that depend on the help of complement, as in antibody-dependent cellular cytotoxicity (ADCC) (Gorter and Meri, 1999). This can be circumvented by the use of bispecific antibodies that bind to CD55 and a tumor-associated molecule, thereby enhancing C3 binding and cell lysis (Blok et al, 1998).

Furthermore, there is some evidence that CD55 and other GPI-linked molecules (CD14, CD24, CD59) might be involved in apoptotic processes (Devitt et al, 1998). A participation of CD55 in apoptosis was observed with regard to human polymorphonuclear leukocytes (PMN), in which a reduced expression of CD55, together with CD59, is closely related to the appearance of apoptotic morphology (Shapiro et al, 1994). In paroxysmal nocturnal hemoglobinuria, a genetically determined hematopoietic stem cell disorder that results in the absence of GPI-linked molecules, including CD55, the cells are also protected from apoptosis when induced by ionized irradiation (Brodsky et al, 1997).

A clear association of CD55 with apoptosis has been shown with the human monoclonal antibody SC-1. This antibody reacts with a carbohydrate residue on a new isoform of GPI-linked CD55 (subsequently named $CD55^{SC-1}$), and induces tumor-cell–specific apoptosis in vitro and in vivo. The $CD55^{SC-1}$ isoform is overexpressed on gastric carcinoma cells (Hensel et al, 1999) and has a molecular weight of approximately 82 kd.

In this paper, we show that stomach carcinoma cells express two different forms of CD55/DAF on the cell surface: the normal 70 kd isoform that protects against complement, and in addition, the new $CD55^{SC-1}$ apoptosis receptor. Furthermore, we show new phenomena associated with SC-1–induced apoptosis, focusing on membrane-associated and cytoplasmic events.

Results

Expression of $CD55^{WT}$ and $CD55^{SC-1}$ on Different Cells

To investigate the expression of the CD55 isoforms biochemically, membrane extracts from the stomach cancer cell line 23132 were blotted with a commercial anti-CD55 antibody and with antibody SC-1. The cervix carcinoma cell line HeLa served as a control for the 70-kd form of CD55. As seen in Figure 1a, two different molecules appear on the stomach cancer cells: a strongly stained 70-kd and a weakly stained 82-kd form. On HeLa cells next to the 70-kd CD55 protein, there is a very strong stained 60-kd band, which seems to be a lower weight isoform of $CD55^{WT}$. With antibody SC-1, only the 82-kd band identified as

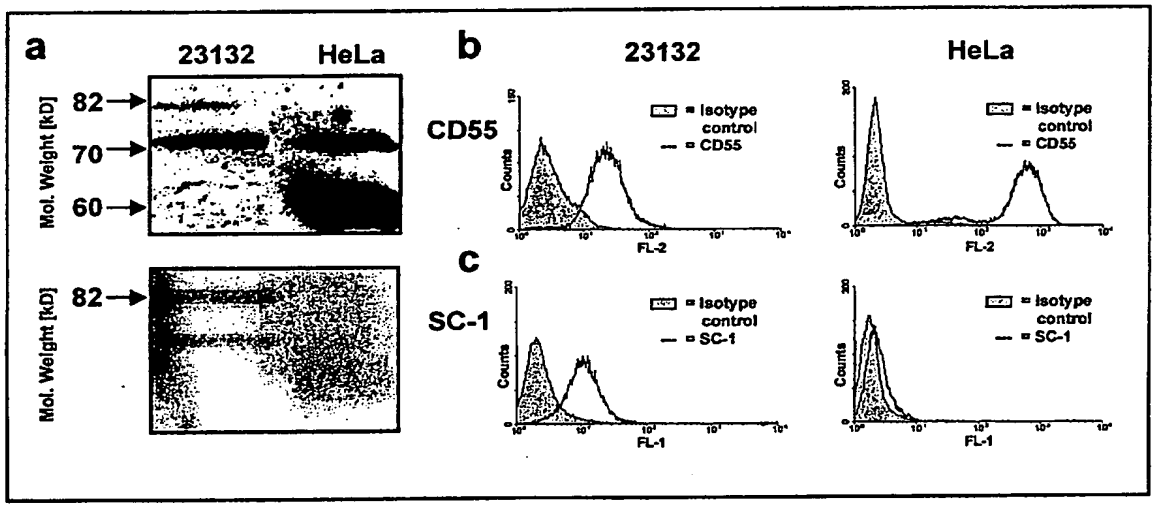

Figure 1.

Expression of CD55 wild-type ($CD55^{WT}$) and SC-1-specific CD55 isoform ($CD55^{SC-1}$) on different cells. The expression of both CD55 isotypes were tested by Western blot and FACS analysis. For Western blot analysis, membrane extracts from 23132 and cervix carcinoma cell line (HeLa) tumor cells were run on SDS gels and blotted on nitrocellulose filters and either stained with anti-CD55 antibody or with SC-1. For FACS analysis, cells were additionally stained with mouse or human isotype-matched control antibodies. a, HeLa cells express two isoforms of CD55 of approximately 60 and 70 kd, whereas in 23132 cells the 70-kd and the SC-1–specific 82-kd form were detectable. SC-1 staining was not observable in HeLa cells, whereas staining of the 82-kd $CD55^{SC-1}$ proteins was visible in the cell line 23132. Staining of additional protein is due to unspecific cross-reaction as described earlier. b, FACS analysis with anti-CD55 shows binding to cell lines 23132 and HeLa compared with the isotype-matched control. c, On cell line 23132, SC-1 shows binding compared with the isotype-matched control, whereas there is no binding found on HeLa cells.

$CD55^{SC-1}$ is stained on stomach cancer cells, whereas there is no reaction on HeLa cells. The second stained band is du to cross-reaction with cytoplasmic Lupus Ku autoantigen described previously (Hensel et al, 1999).

To confirm the Western blot analysis, we performed flow cytometry by staining both cell lines with SC-1 or anti-CD55 antibody. The flow cytometry clearly shows that the anti-CD55 antibody binds to the 23132 and HeLa cells (Fig. 1b), whereas antibody SC-1 clearly binds to 23132 cells, but not to HeLa cells (Fig. 1c).

Internalization of $CD55^{SC-1}$ on Cell Line 23132 after Induction of Apoptosis

We further examined the membranous expression of $CD55^{WT}$ and $CD55^{SC-1}$ in cell line 23132 after the induction of apoptosis with 40 μg/ml of SC-1 by flow cytometry with anti-CD55 and SC-1 antibodies. Flow cytometry shows that $CD55^{SC-1}$ expression remains unchanged for 24 hours, whereas there is a clear decrease in staining with antibody SC-1 after 48 hours. After 72 hours, SC-1 staining is restored (Fig. 2, a to d). In contrast, expression of $CD55^{WT}$ remains stable for the measured period (Fig. 2, e to h). Because both CD55 isoforms arise by posttranslational modification and are not different gene products, these differences must occur as a result of the internalization of $CD55^{SC-1}$ after the binding of SC-1. These data were confirmed by cytospin preparations (data not shown). Controls were performed by staining cells with 40 μg/ml of chrompure human IgM for the indicated times.

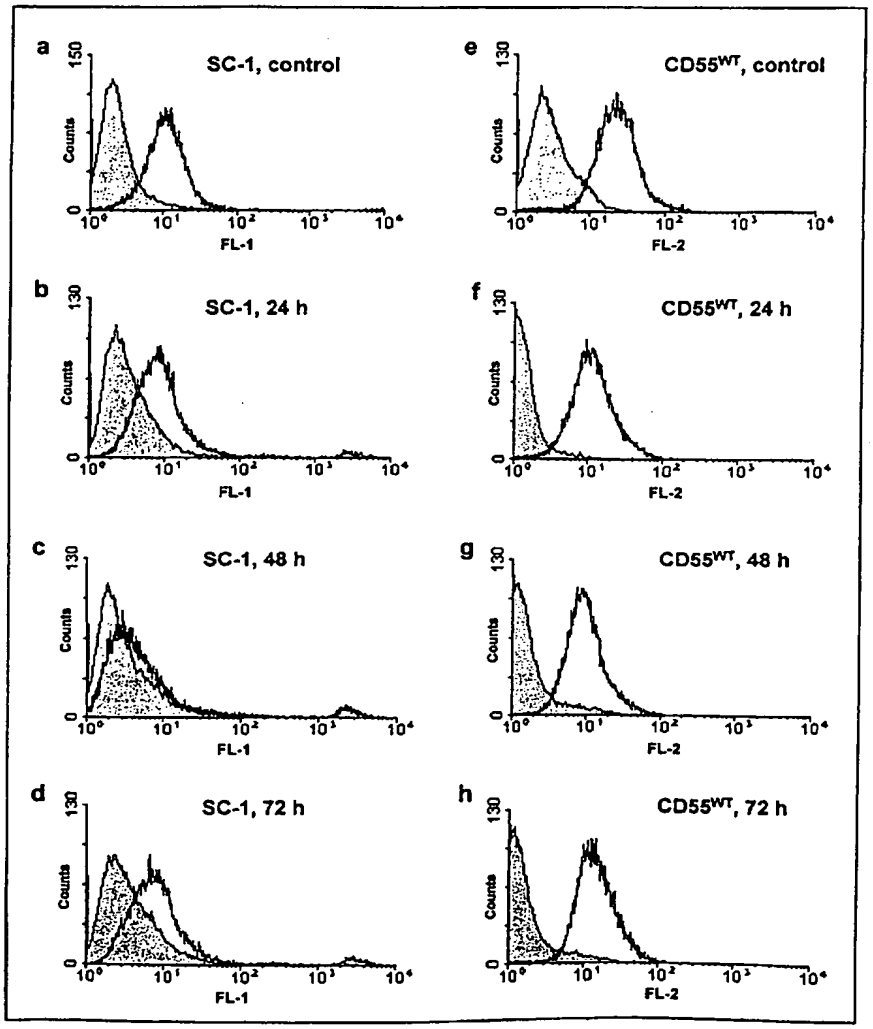

Figure 2.
Expression of $CD55^{SC-1}$ and $CD55^{WT}$ after induction of apoptosis by SC-1. Cells were incubated for the indicated times with 40 μg/ml of SC-1, and after trypsinization, cells were analyzed for expression of $CD55^{SC-1}$ (a–d) and $CD55^{WT}$ (e–h) by flow cytometry. Additionally, cells were stained with mouse or human isotype-matched control antibodies. *Gray area* indicates isotype-matched controls; *black line* indicates cells stained by SC-1 and $CD55^{WT}$.

Cleavage of Cytokeratin 18

The degradation of apoptotic pithelial cells is accompanied by the proteolytic cleavag of cytokeratin 18 (Caulin t al, 1997). We investigated th cleavage of cytokeratin 18 in cell line 23132 after SC-1–induced apoptosis using the M30 CytoDeath kit. Cytokeratin 18 cleavage starts after 24 hours, as determined by immunohistochemical staining (Fig. 3b). After 48 hours, cleavage is completed, and apoptotic bodies are released from the cells (Fig. 3c). Figure 3, d to f, shows that approximately 30% of the cells are undergoing apoptosis. Controls were performed by treating cells for equal periods with 40 μg/ml of chrompure human IgM antibody without inducing cytokeratin 18 cleavage (data not shown).

Effect of Inhibition of Caspase-6 and Caspase-3

The cleavage of cytokeratin 18 indicates the involvement of caspase-6 in the apoptotic process induced by SC-1. To confirm the participation of caspase-6 in the degradation process, the effect of caspase-6 inhibitor Val-Glu-Iso-Asp-aldehyde (VEID-CHO) on apoptosis was investigated. Cells were incubated overnight with increasing concentrations of the inhibitor VEID-CHO, followed by incubation with 40 μg/ml of SC-1 for 24 hours. The effect of inhibition of caspase-6 was measured using CellDeath ELISA. Surprisingly, low amounts of inhibitor increased apoptotic cell death, whereas high concentrations clearly inhibited apoptosis (Fig. 4a), indicating that recruitment of caspase-6 is necessary for SC-1–induced apoptosis.

In a recent publication (Hensel et al, 1999), we showed that caspase-3 is activated in SC-1–induced apoptosis. Because of the surprising results obtained from the inhibition of caspase-6, we investigated whether the inhibition of caspase-3 by Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) has a comparable effect on SC-1–induced apoptosis. Here, we also found an increase in apoptotic cell death with increasing concentrations of inhibitor. Cells incubated with 500 nM of DEVD-CHO showed an absorption approximately three times higher, whereas incubation with inhibitor without SC-1 did not have any effect on spontaneous apoptosis (Fig. 4b).

Molecular Analysis of SC-1–Induced Apoptosis

The occurrence of poly(ADP-ribose)polymerase (PARP) cleavage was investigated by Western blot analysis, using whole cell extracts from SC-1–induced cells and murine anti-PARP antibody. In five indepen-

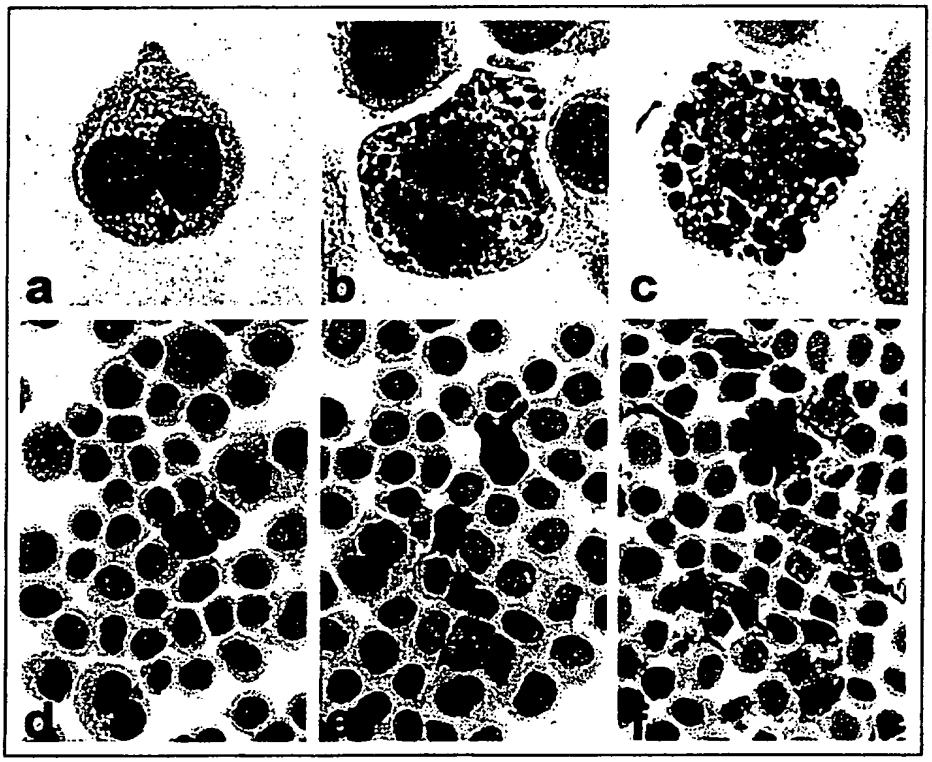

Figure 3.
Cleavage of cytokeratin 18 in SC-1–induced apoptosis. Immunohistochemical staining of cytospins reveals that 24 hours after the induction of apoptosis, cleavage of cytokeratin 18 starts (b), and after 48 hours, apoptotic bodies are released from the cells (c). In Panel a, a nonapoptotic cell is shown (magnification, ×400). The overview shows a low amount of apoptosis in uninduced cells (d), whereas SC-1–induced apoptosis begins after 24 hours (e). After 48 hours, approximately 30% have undergone apoptosis as shown by cytokeratin 18 cleavage (f) (magnification, ×400).

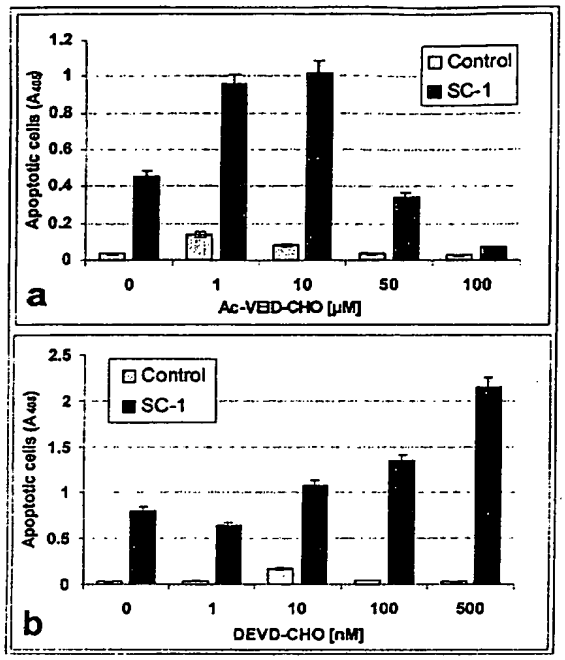

Figure 4.
Inhibition of caspase-6 and caspase-3 by specific inhibitors. Cells were incubated overnight with increasing amounts of caspase inhibitor and then for another 24 hours with 40 μg/ml of SC-1. Apoptotic cells were determined by CellDeath ELISA. a, Effect of caspase-6 inhibitor VEID-CHO. Low concentrations of inhibitor increase apoptosis, whereas high concentrations clearly inhibit apoptosis. b, Effect of caspase-3 inhibitor DEVD-CHO. An increase in apoptotic cells with increasing concentrations of inhibitor is observable, whereas incubation with only DEVD-CHO does not show any effect.

dent assays, there was no observable PARP cleavage, which would have been marked by the occurrence of an 85-kd cleavage product (Cosio et al, 1994) (Fig. 5a). The induction of apoptosis was confirmed by measurement of caspase-3 and caspase-8 activity as published earlier (Hensel et al, 1999), and the functionality of the anti-PARP antibody was confirmed by Western blot analysis with lysates of Fas-induced cells (data not shown).

To investigate the changes in the cell cycle after the induction of apoptosis, the expression of topoisomerase IIα was tested by Western blot analysis. Topoisomerase IIα is a key enzyme in the cell cycle by virtue of being involved in DNA replication (Schmitt et al, 1999). Therefore, the reduced expression of topoisomerase IIα after SC-1–induced apoptosis indicates cell-cycle arrest for at least a fraction of the cells (Fig. 5b).

The transcription factor c-myc has been shown to be involved in various apoptotic processes or to induce apoptosis by transfection in cells (Berstad and Brandtzaeg, 1998). Because c-myc is not involved in all apoptotic processes, we investigated the expression pattern of c-myc after SC-1–induced apoptosis. A clear increase in c-myc expression was found 5 minutes after induction of apoptosis, followed by a decrease after 1 hour (Fig. 5b). Control for nonspecific effects induced by human IgM was performed by incubating cells with 40 μg/ml of chrompure human IgM antibody without any changes in the expression pattern of any of the proteins described above (data not shown).

Relation between Intracellular Ca^{2+} Concentration and Apoptosis

To investigate whether the induction of apoptosis by SC-1 is accompanied by changes of the intracellular calcium concentration $[Ca^{2+}]_i$, we measured the $[Ca^{2+}]_i$ of cell line 23132 after induction with SC-1 and control antibody (chrompure human IgM), using an Axiovert TV microscope. Approximately 1 minute after the addition of SC-1 antibody, a significant increase of $[Ca^{2+}]_i$ was observed, whereas the control antibody did not have any effect on $[Ca^{2+}]_i$ (Fig. 6a).

To investigate the role of $[Ca^{2+}]_i$ in detail, cells were incubated for 3 hours with increasing amounts of

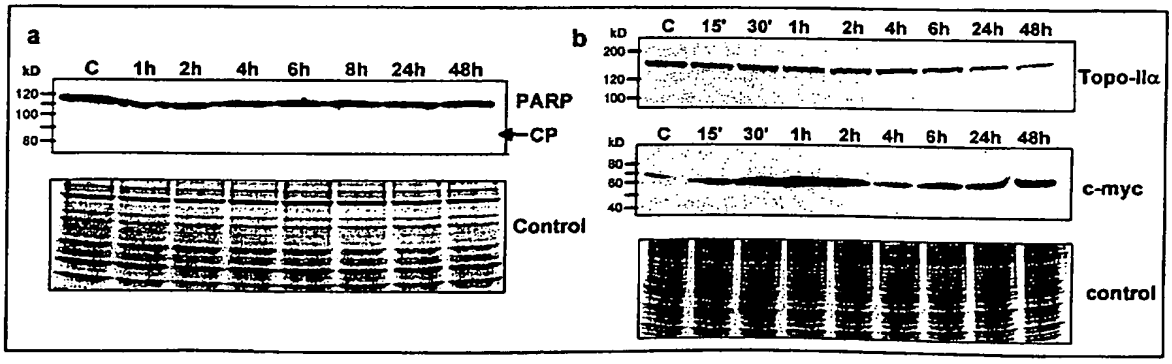

Figure 5.
Western blot analysis of SC-1–induced cells. Cell line 23132 was induced with 40 μg/ml of SC-1 for the periods indicated above, and then whole cell lysates and Western blots were prepared. Coomassie-stained gels shown below the blots were used as controls for equal protein concentrations loaded on lanes. a, Determination of poly(ADP-ribose)polymerase (PARP) cleavage by anti-PARP antibody. *CP* indicates expected size of PARP cleavage product. Cleavage of PARP was not detectable at all. b, Staining with anti-topoisomerase IIα antibody as marker for cellular proliferation. A reduced expression of topoisomerase IIα could be observed after SC-1–induced apoptosis. Expression of c-myc detected by staining with anti-c-myc antibody. Five minutes after induction of apoptosis, an increased c-myc expression was found, followed by a decrease after 1 hour.

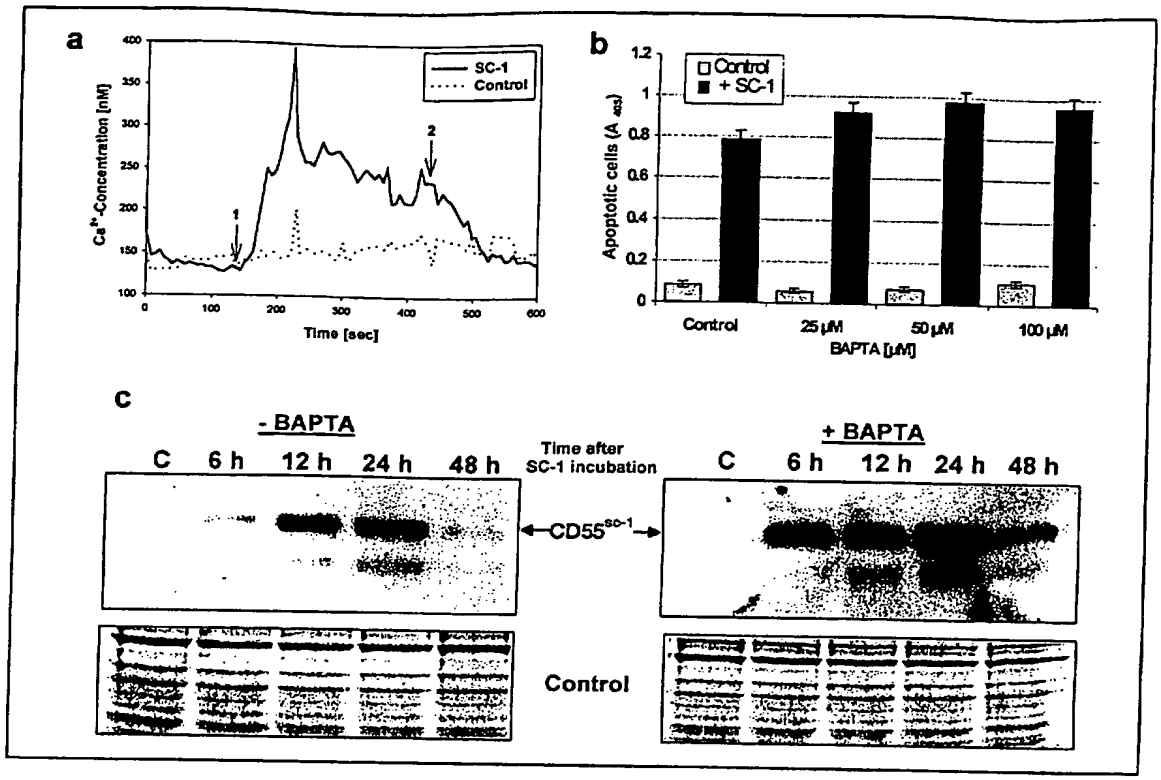

Figure 6.

Participation of $[Ca^{2+}]_i$ in the apoptotic process. a, Measurement of $[Ca^{2+}]_i$ after SC-1 induction: Cells were washed with Ringer solution, and, at Point 1, SC-1 (40 µg/ml) or control antibody chrompure human IgM (40 µg/ml), diluted in Ringer solution, was added. At Point 2, cells were washed with Ringer solution. A 2.7-fold increase in intracellular Ca^{2+} concentration was observed after approximately 50 seconds of induction with SC-1. b, Effect of Ca^{2+}-Chelator, 1, 2-bis(2-aminophenoxy)ethane-N, N,N',N'-tetraacetic acid (BAPTA) on apoptosis: Cells were preincubated for 90 minutes with increasing amounts of BAPTA, followed by incubation with 40 µg/ml of SC-1 for 24 hours. Apoptotic cells were determined using CellDeath ELISA. An increasing amount of BAPTA had no effect on cell survival after induction of SC-1–induced apoptosis. Treatment with increasing amounts of BAPTA without SC-1 did not show any effect. c, Effect of Ca^{2+}-Chelator BAPTA on $CD55^{SC-1}$ expression: Cell line 23132 was induced with 40 µg/ml of SC-1 in the presence or absence of BAPTA for the periods indicated above, and then membrane lysates and Western blots were prepared and stained with SC-1. Coomassie-stained gels show loading of equal protein concentrations on each lane. A clear increase in $CD55^{SC-1}$ expression with different kinetic was visible in both experiments. Staining of additional protein is due to an unspecific cross-reaction with the Ku70 autoantigen as described earlier.

Ca^{2+}-chelator, 1, 2-bis(2-aminophenoxy)ethane-N, N,N',N'-tetraacetic acid (BAPTA), followed by incubation with 40 µg/ml of SC-1 for 24 hours. The number of apoptotic cells was determined by CellDeath ELISA. Increasing the amount of BAPTA does not have any effect on cell survival, as can be seen in non-SC-1–induced cells. Furthermore, there is not a decrease in apoptotic cells after induction of apoptosis with an increasing amount of BAPTA (Fig. 6b). These data indicate that the intracellular Ca^{2+} concentration is not involved in the regulation of SC-1–induced apoptosis. However, as shown by immunohistochemical studies on cytospins, $[Ca^{2+}]_i$ might be involved in membrane expression of $CD55^{SC-1}$ and, therefore, may only indirectly be responsible for apoptosis. Neither an increase of membrane expression nor an upsurge of $CD55^{SC-1}$ is detected if $[Ca^{2+}]_i$ is blocked by BAPTA, as seen after SC-1 binding (data not shown).

To further explore the difference in expression of $CD55^{SC-1}$ in untreated and BAPTA-treated cells after SC-1-induction, Western blot analysis was performed with membrane extracts from SC-1-induced cells. An increased expression of $CD55^{SC-1}$ was observed beginning 6 hours after induction of apoptosis and lasting up to 48 hours. However, in uninduced cells $CD55^{SC-1}$ was not detectable because of the low sensitivity of the SC-1 antibody in Western blot analysis. In cells pretreated with BAPTA, an even faster increase in $CD55^{SC-1}$ expression is observed, which also is detectable for up to 48 hours (Fig. 6c). Interestingly, during this apoptotic process, an increase in the expression of the cytoplasmic 70-kd protein, which cross-reacts with SC-1 and was formerly identified as Ku70 autoantigen (Hensel et al, 1999), can also be seen in Western blot analysis.

Discussion

The SC-1/DAF pathway for tumor-specific apoptosis is new and unique in several aspects. The 82-kd $CD55^{SC-1}$ isoform is specifically overexpressed on gastric carcinoma cells, together with the "normal"

70-kd isoform of $CD55^{WT}$. SC-1 apoptosis induces a strong up-regulation of the specific 82-kd $CD55^{SC-1}$ isoform, followed by its internalization. In contrast, the 70-kd isoform of $CD55^{WT}$, which is also pr sent on stomach cancer cells is not up-regulated or internalized. The apoptotic event is accompanied by cleavage of cytokeratin 18, which indicates the involvement of caspase-6. Caspase-6 is necessary for SC-1–induced apoptosis, because the inhibition by high concentration VEID-CHO suppresses apoptosis, whereas the inhibition of caspase-3 by DEVD-CHO leads to increased apoptosis. Cleavage of PARP is not observable during SC-1–induced apoptosis, which indicates a pathway different from the one shown for Fas. The apoptotic activity of $CD55^{SC-1}$ is not dependent on $[Ca^{2+}]_i$, because the calcium blocker BAPTA does not reduce apoptosis, but BAPTA has some inhibitory effect on the transport of $CD55^{SC-1}$ to the membrane.

Our data show that two isoforms of CD55 are expressed in stomach carcinoma: the ubiquitously distributed 70-kd $CD55^{WT}$ and, additionally, the specific 82-kd $CD55^{SC-1}$ isoform. Interestingly, both CD55 isoforms show a different regulation after induction of apoptosis. Since there were not any differences found in the transcripts of CD55 in cell line 23132 compared with other published wild-type sequences (Hensel et al, 1999), regulation of the two isoforms must be due to posttranslational events like differential glycosylation, which will have to be examined more closely. The up-regulation of apoptosis-inducing receptors during apoptosis is a feature of these receptors unobserved so far, and the genetic background of this observation has to be further investigated. This up-regulation might be coupled to the function of CD55 in the protection of cells from autologous complement (Cheung et al, 1988). Up-regulation of CD55 by various factors has been observed in noncancerous cells, eg, in endothelial cells (ECs) or human glomerular cells. This up-regulation can be induced by incubating ECs with the membrane attack complex (MAC) and by incubation with anti-CD55 antibodies or various cytokines, such as tumor necrosis factor (TNF)-α or interferon (IFN)-γ (Mason et al, 1999). Increased expression of CD55 can also be induced by activation of terminal complement compounds (C8 and/or C9) on human glomerular cells (Cosio et al, 1994). It might be suggested that this up-regulation of CD55 results in an elevated resistance to the attack of autologous complement.

So far the inducibility of CD55 expression on tumor cells is not well investigated. An increased expression of the complement regulators CD46, CD55, and CD59 has been shown immunohistochemically on gastrointestinal tumors and might also have a function in the protection of cells from complement attack (Berstad and Brandtzaeg, 1998; Schmitt et al, 1999). Our data show for the first time that CD55 is inducible in stomach cancer cells and leads us to suggest that SC-1 partially mimics complement attack. The higher expression of $CD55^{SC-1}$ is followed by the disappearance of $CD55^{SC-1}$ from the cell membrane, as demonstrated by Western blot analysis and flow cytometry. This increase might be a protection mechanism for the cell against the apoptotic mechanism of the SC-1 antibody. This is supported by the finding that the disappearance of $CD55^{SC-1}$ is observ d in nearly all cells, wher as apoptosis, shown by cleavage of cytokeratin 18, is only visible in approximately 30% of the cells. Further studies will show whether the disappearance of CD55 from the membrane is related to the protection of cells against apoptosis.

The apoptotic process is accompanied by limited proteolysis of cellular proteins by the caspase family of cysteine proteases (Alnemri et al, 1996), which are mediators of apoptotic cell death (Martin and Green, 1995). One target of these proteases is cytokeratin 18, a major component of the intermediate filament of simple epithelial cells and tumors derived from such cells (Caulin et al, 1997; Schaafsma et al, 1990). So far, the cleavage of cytokeratin 18 has been observed in chemically induced (etoposide) or UV-light–induced apoptosis. Our data show that cleavage of cytokeratin 18 also occurs in SC-1–induced apoptosis. This cleavage is visible in 30% of the cells and increases for up to 72 hours after induction of apoptosis, indicating that SC-1–induced apoptosis is slower than chemical- or UV-light–induced apoptosis. Furthermore, this data shows that caspase-6, which performs the initial cleavage of cytokeratin 18 into 26-kd and 22-kd fragments (Caulin et al, 1997), must be a participant in SC-1–induced apoptosis. The necessity of the participation of caspase-6 was shown by the inhibition of caspase-6 with VEID-CHO, which reduces the amount of apoptotic cells when using higher concentrations of caspase inhibitor. The increase in apoptotic cell death when using low concentrations (1–10 μM) of VEID-CHO and the increase in cell death with increasing amounts of caspase-3 inhibitor DEVD-CHO indicate a number of differences from apoptosis pathways previously described. This might be due to the bypassing of the inhibited caspases by means of other caspases. These differences from other apoptosis pathways are consistent with the fact that PARP cleavage does not occur in SC-1–induced apoptosis. PARP is a 116-kd protein that detects and binds to DNA strand breaks and is involved in DNA repair (Casciola-Rosen et al, 1996). PARP is cleaved specifically by caspase-3 after its activation by proteolytic cleavage (Lazebnik et al, 1994). For that reason, cleavage of PARP is widely used as a marker for the induction of apoptosis (Oliver et al, 1998). Whether this observation is due to the low activation of caspase-3 (Hensel et al, 1999) needs to be further investigated. However, caspase-3 does not seem to play any role in SC-1 apoptosis, and this provides further evidence that cleavage of PARP is not obligatory for induction of apoptosis. Recently, it was shown that PARP−/− fibroblasts expressing mutant uncleavable PARP are sensitive to CD95 apoptosis, but with a delayed cell death (Oliver et al, 1998).

Slow kinetics are also visible in SC-1–induced apoptosis, as confirmed by staining with M30 CytoDeath antibody and the slow decrease in expression of topoisomerase IIα. The involvement of the cell-cycle regulator c-myc in apoptotic processes has been

shown in various experimental systems (Packham and Cleveland, 1995). Activation or overexpression of c-myc induces apoptosis, whereas cells with reduced c-myc expression seem to be resistant to various apoptotic stimuli (Dong et al, 1997). The up-regulation of c-myc expression in SC-1–induced apoptosis leads us to propose that SC-1 apoptosis mediated by $CD55^{SC-1}$ is also dependent on c-myc. It has been shown that c-myc–dependent apoptosis induces cleavage of PARP and activation of caspase-3. Because Ca^{2+} is also a regulator of some apoptotic processes (Yoshida et al, 1997), we investigated the effect of changes in the intracellular Ca^{2+} concentration on SC-1–induced apoptosis, and a rapid increase in the $[Ca^{2+}]_i$ was found. This increase in $[Ca^{2+}]_i$ is not related to the apoptosis event, because apoptosis cannot be inhibited by the intracellular Ca^{2+} blocker BAPTA. Yet the differences in the expression pattern shown by Western blot analysis indicate that $[Ca^{2+}]_i$ is involved in the regulation of CD55 expression. Also, membrane translocation of CD55 is regulated by Ca^{2+}, because, after the incubation of cells with BAPTA and induction of apoptosis, an increase in membranous staining is not visible (data not shown). Next to an increase of $CD55^{SC-1}$, we found an increase of a second protein of approximately 70 kd, which was identified as the Ku70 autoantigen (Hensel et al, 1999) and shows some cross-reaction with the SC-1 antibody. It was shown recently that this protein is involved in the apoptotic process. There was an increase of Ku70 expression found after induction of apoptosis by ionizing radiation in human lung carcinoma cell lines (Brown et al, 2000). Therefore, our data lead us to believe that Ku70 also plays a role in SC-1–induced apoptosis.

Complement-inactivating receptors have been shown to be overexpressed on a variety of tumor cells and represent ideal targets for therapeutic approaches. However, our data have shown that the expression is not stable but, rather, is strongly variable. This limits the use of antibody-mediated cell lysis and vaccination for therapy. For ADCC, high amounts of CD55 inactivate the complement, which is needed by the therapeutical antibodies (Riethmüller et al, 1994), and low expression reduces immunological processes (Durrant et al, 2000).

The induction of apoptosis by the IgM antibody SC-1 is relatively independent of the number of CD55 receptors. The reason for this is that, shortly after the binding of SC-1 to CD55, the cell increases its amount of CD55 on the surface to protect itself against a presumptive complement attack. This, ironically, does not help the cell to survive but, instead, enhances cell death.

We do not know why stomach cancer cells express two isoforms of CD55 with different glycosylation patterns. It could provide an advantage in protection against autologous complement, and at least it does not have any negative selective effect in vivo. The amounts of circulating antibodies like SC-1 are generally too low to become dangerous for the tumor. However, it would be interesting to examine tissues from other tumors for similar coexpression, and possible targets similar to those on stomach cancer cells could be detected.

Apoptosis is the most effective and safest way to remove tumors from the organism. So far, many apoptosis receptors are known, but none of them are tissue- or cell-specific, or at least overexpressed on tumor cells. The SC-1/CD55 apoptosis mechanism is, so far, unique, but it may be assumed that the search for new tumor-related apoptosis receptors on other malignant cells is likely to succeed, if one begins by looking for mechanisms first, and then for corresponding molecules. This will result in more promising targets for cancer therapy.

Material and Methods

Cell Culture

For all assays, the established stomach adenocarcinoma cell line 23132 (Vollmers et al, 1993) was used. Cells were grown to subconfluency in RPMI-1640 (PAA, Vienna, Austria), supplemented with 10% FCS and penicillin/streptomycin (both 1%). For the assays described here, cells were detached with trypsin/EDTA and washed twice with PBS before use. The human hybridoma cell line SC-1 was grown in serum-free RPMI-1640 medium (PFHM-II; Life Technologies-Gibco BRL, Karlsruhe, Germany), using miniPerm Bioreactors (InVitro Systems & Services, Osterode, Germany).

Purification of the SC-1 Antibody

The human monoclonal antibody was purified from mass cultures, using cation exchange chromatography followed by gel filtration, as described elsewhere (Vollmers et al, 1998a).

Flow Cytometry

The cell lines 23132 and HeLa were used for the analysis of $CD55^{SC-1}$ and $CD55^{WT}$ receptor expression. Cells were grown to subconfluency in complete medium, and then purified SC-1 was added to a final concentration of 40 μg/ml for the indicated periods. As a control, cells were incubated in RMPI-1640 medium with 10% FCS without SC-1 antibody. Cells were harvested after 24, 48, and 72 hours by detaching with Trypsin/EDTA. The cells were subsequently incubated on ice with SC-1, anti-CD55 antibody (clone 143-30, DPC Biermann, Bad Nauheim, Germany), and human (Chrompure human IgM; Dianova, Hamburg, Germany) or mouse isotype-matched (mouse IgG1; Pharmingen, Heidelberg, Germany) control antibodies for 15 minutes. This was followed by incubation with a FITC-labeled rabbit anti-human IgM antibody (Dianova) or an R-phycoerythrin-labeled donkey anti-mouse IgG (Dianova), respectively, for 15 minutes on ice. Antibodies were optimally diluted in PBS containing 0.01% sodiumazide. Cells were analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, California).

Immunohistochemical Staining of Cytospin Preparations

Subconfluently grown cells were incubated with purified SC-1 antibody diluted to 40 μg/ml in complete growth medium and incubated for up to 48 hours. Adherent and detached cells were collected after the prescribed times, centrifuged, and resuspended in complete growth medium. After counting cells, cytospins were prepared and air-dried at room temperature overnight. Cytospins were blocked with BSA (15 mg/ml) diluted in PBS for 30 minutes. The cytospins were incubated for 1 hour with M30 CytoDeath antibody (Roche Biochemicals, Mannheim, Germany) and washed for 30 minutes in PBS, followed by incubation with peroxidase-labeled rabbit anti-mouse conjugate (Dako, Glostrup, Denmark) diluted 1:25 in PBS/BSA. After washing for 30 minutes with PBS, staining was performed with diaminobenzidine (0.05%)-hydrogen peroxide (0.02%) for 10 minutes at room temperature. The reaction was stopped under running tap water, and sections were counterstained with hematoxylin.

Inhibition of Caspases and Apoptosis Assay

Cells were preincubated for 24 hours with the indicated concentrations of caspase inhibitors. Then the purified SC-1 antibody was added to the final concentration of 40 μg/ml, and plates were incubated for a further 24 hours. Apoptosis was detected using the CellDeath Detection^{plus} kit (Roche Biochemicals) following the manufacturer's protocols.

Preparation of Cell Lysates after Induction with SC-1

Cell line 23132 was grown to subconfluency on 100-mm cell-culture plates. Then the SC-1 antibody was added to a final concentration of 30 μg/ml and incubated for the periods indicated. After incubation, culture plates were washed once with PBS, and subsequently cells were directly lysed with SDS buffer (50 mM Tris/Cl, pH 6.8; 10 mM DTT; 2% [w/v] SDS; 10% [v/v] glycerol). Cell lysates were collected with a rubber policeman.

For preparation of membrane proteins, harvested cells were resuspended in hypotonic buffer (20 mM HEPES, 3 mM KCl, 3 mM $MgCl_2$), incubated on ice (15 minutes), and sonicated (5 minutes), and the nuclei were pelleted by centrifugation (10,000 ×*g*, 10 minutes). The membranes were pelleted by centrifugation (100,000 ×*g*, 30 minutes) and resuspended in membrane lysis buffer (50 mM HEPES, pH 7.4; 0.1 mM EDTA; 1 M NaCl; 10% glycerol; and 1% Triton X-100). Complete protease inhibitor (Roche Biochemicals) was added to all solutions.

Gel Electrophoresis and Blotting

SDS-PAGE under reducing conditions and Western blotting of proteins were performed using standard protocols as described elsewhere (Vollmers et al, 1997). In brief, blotted nitrocellulose membranes were blocked with PBS containing 0.05% (v/v) Tween-20 (except for SC-1) and 5% (w/v) low-fat milk powder, followed by a 1-hour incubation with primary antibody. The antibodies were used in the indicated dilutions: SC-1, 10 μg/ml; mouse anti-topoisomerase IIα, 1:1000 (Neomarkers, Baesweiler, Germany); anti-c-myc, 1:1000; anti-CD55, 1:1000 (Santa Cruz, Heidelberg, Germany); and anti-PARP, 1:1000 (Pharmingen). The secondary antibodies (peroxidase-coupled rabbit anti-mouse IgG or rabbit anti-goat antibody [Dianova]) were detected with the SuperSignal chemiluminescence kit from Pierce (KMF, St. Augustin, Germany).

Measurement of Intracellular Free Calcium $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using the Ca^{2+}-sensitive dye, Fura 2-AM, as described (Grynkiewicz et al, 1985). In brief, cells were incubated with Ringer solution (122.5 mM NaCl; 5.4 mM KCl; 1.2 mM $CaCl_2$; 0.8 mM $MgCl_2$; 1 mM NaH_2PO_4; 5.5 mM glucose; 10 mM HEPES, pH 7.4) containing Fura 2-AM in a final concentration of 5 μM for 15 minutes. After rinsing, the coverslips were mounted on the stage of an inverted Axiovert 100 TV microscope (Zeiss, Jena, Germany; magnification, ×400). The fluorescence signal was monitored at 500 nm with excitation wavelengths alternating between 334 and 380 nm, using a 100 Watt xenon lamp and an automatic filter change device (Zeiss). Filter change and data acquisition were controlled by Attofluor software (Zeiss). $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al (1985), with a dissociation constant of 225 nM. The maximum and minimum fluorescence ratios (R_{max} and R_{min}) were measured after addition of the calibration solutions. R_{max} was measured in the presence of Ringer solution containing 3 mM Ca^{2+} and 1 μM ionomycin, and R_{min} was measured in the presence of Ca^{2+}-free Ringer solution containing 3 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) and 1 μM ionomycin.

Inhibition of Intracellular Calcium Release

Cells were washed once with PBS and incubated for 3 hours with the indicated concentrations of BAPTA diluted in complete growth medium. Then purified SC-1 antibody was added to a final concentration of 40 μg/ml. As a control, the same cells were incubated without SC-1. Cells were incubated in a humidified incubator for an additional period of time as indicated and then used for either preparation of membrane extracts or performing CellDeath ELISA as described below, or they were fixed with 3% paraformaldehyde for morphological analysis. Cell-culture plates were analyzed for morphologic changes with the aid of a light microscope.

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